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Si aucun titre n'est indiqué se référer à la description.)

New antibodies directed against hepatitis C virus, compositions of particles
liable to be recognized by said antibodies, and pharmaceutical compositions
containing the same

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NEW ANTIBODIES DIRECTED AGAINST HEPATITIS C VIRUS, COMPOSITIONS OF PARTICLES LIABLE TO BE RECOGNIZED BY SAID ANTIBODIES, AND PHARMACEUTICAL COMPOSITIONS CONTAINING THE SAME

5

The present invention relates to new conformational antibodies directed against HCV and more particularly to monoclonal antibodies. The invention also relates to compositions of particles liable to be recognized by said antibodies, and to pharmaceutical compositions containing them. The invention also relates to HCV enveloped subviral particles or purified HCV enveloped complete viral particles, and the processes for preparing them.

Hepatitis C Virus (HCV) infection is a major cause of chronic hepatitis and cirrhosis and may lead to hepatocellular carcinoma. With about 200 million people worldwide chronically infected with HCV, this disease has emerged as a serious global health problem. HCV is an enveloped RNA virus belonging to the genus *Hepacivirus* of the *Flaviridae* family. Its genome is a 9.6-kb single-stranded RNA of positive polarity with a 5' untranslated region (UTR) that functions as an internal ribosome entry site, a single open reading frame encoding a polyprotein of approximately 3,000 amino acids and a 3'UTR (Bartenschlager *et al.*, 2000). This polyprotein is co- and post-translationally cleaved by host cell peptidases to yield the structural proteins including the core protein and the envelope glycoproteins E1 and E2, and by viral proteases to generate the non-structural proteins (NS) 2 to 5B (Bartenschlager *et al.*, 2000). By analogy to related positive-strand RNA viruses, replication occurs by means of a negative-strand RNA intermediate and is catalyzed by the NS proteins, which form a cytoplasmic membrane-associated replicase complex.

The low levels of HCV particles present in patient plasma samples and the lack of a cell culture system supporting efficient HCV replication or particle assembly have hampered the characterization of the glycoproteins associated with the virion. The current knowledge on HCV envelope glycoproteins is based on cell culture transient-expression assays with viral or non viral expression vectors. These studies have shown that the E1 and E2 glycoproteins interact to form complexes (reviewed in Dubuisson, 2000). In the presence of nonionic detergents, two forms of E1E2 complexes are detected: an E1E2 heterodimer stabilized by noncovalent interactions and heterogeneous disulfide-linked aggregates, which are considered to represent misfolded complexes. Previously, envelope glycoprotein-specific antibodies have been obtained by immunization with synthetic peptides or recombinant antigens. A

conformation sensitive E2-reactive monoclonal antibody (mAb) (H2), which recognizes noncovalently-linked E1E2 heterodimers considered as the native prebudding form of the HCV glycoprotein heterodimer, however does not react with serum-derived HCV RNA-positive particles (Deleersnyder *et al.*, 1997). Furthermore, WO 92/07001 discloses antibodies which have been prepared by immunization of mice with a preparation of HCV particles extracted from infected chimpanzees, however these antibodies have not been tested on natural HCV particles (i.e. derived from infected patients). Moreover, WO 00/05266 discloses antibodies prepared from infected patients B cells, however these antibodies have been selected according to their ability to bind to the recombinant E2 protein. Therefore, all these antibodies are of limited use, either for diagnostic purposes, or for therapeutic or prophylactic purposes, as they have been produced or selected with unnatural HCV, or parts thereof, and have not been shown to interact with natural HCV particles. The lack of HCV preparation containing natural enveloped HCV particles in sufficient quantity and concentration, is one of the reason for which antibodies liable to recognize natural HCV particles could not be obtained so far.

Thus, an object of the invention is to provide antibodies which react with natural HCV particles.

Another aspect of the invention is to provide compositions of natural HCV particles, in sufficient quantity and concentration to allow efficient immunization of antibody producing animals.

Another aspect of the invention is to provide specific HCV compositions devoid of infectivity, liable to be used as active substances of pharmaceutical compositions.

Detailed description of the invention

The present invention relates to a conformational antibody capable of specifically binding to the natural HCV viral envelope.

The expression "conformational antibody" designates an antibody which recognizes an epitope having a three-dimensional structure defined by its molecular surrounding.

The expression "specifically binding" means that the antibody binds to an epitope which is found on substantially only one of the elements forming the natural HCV viral envelope, that is to say that there is substantially no binding of the antibody with elements other than the elements forming the natural HCV envelope. For example, the binding specificity of the antibody can be tested by methods well known to the man skilled in the art, such as Western

blotting experiments, wherein biological samples are electrophoretically migrated on a gel, then transferred on a membrane and co-incubated with said antibody, which is then detected by a secondary antibody. Said antibody is said to be "specifically binding" to a target compound contained in said biological samples if substantially all of the electrophoretical bands detected contain the target compound or parts thereof.

"HCV" means "hepatitis C virus", it is in particular described in Choo *et al.* (1989, 1991). HCV particularly comprises RNA, a capsid made of a core protein, and an envelope which comprises lipids and proteins, particularly glycoproteins.

The "HCV viral envelope" is made of lipids and proteins, in particular glycoproteins such as HCV proteins E1 and E2 (Clarke, 1997).

"Natural" means that HCV, or parts thereof, are as found in biological samples and possibly as isolated and if needed as purified from biological samples. Such samples may be blood, plasma or sera, of patients infected by HCV. In particular, "natural" refers to HCV, or parts thereof, which have not been produced by recombinant methods, or by using cell lines or animals, and are different from HCV elements described in Schalich *et al.* (1996), Blanchard *et al.*, (2002) or WO 92/07001 for instance.

The present invention more particularly relates to a conformational antibody capable of specifically binding to the natural HCV E2 protein.

HCV E2 is in particular described in Dubuisson (2000) and Op De Beeck *et al.* (2001). It is produced as a polyprotein (3012 amino acids) which is cleaved to yield E2 (amino acids 384 to 714).

The expression "specifically binding" means that the antibody binds to an epitope, or a part thereof, which is found on substantially only the HCV E2 protein. In particular the antibody can not be detached from the HCV E2 protein in competition tests, wherein the antibody-E2 complex is presented with other proteins.

The expression "natural HCV E2 protein" means that the protein is as found in biological samples of patients infected by HCV, in particular the natural HCV E2 protein is not a recombinant protein.

The present invention relates to a conformational antibody as defined above, capable of neutralizing HCV infections in patients.

"Neutralizing HCV infection" means that the antibody is capable of improving the health of patients infected by HCV, as can be evidenced, for instance, by the diminishing of HCV detected in blood, plasma or sera, or that the antibody is capable of preventing individuals to be infected by HCV

The HCV infection neutralization capability of said antibody can be monitored in model animals, such as chimpanzees or mice, in particular humanized mice, which are chronically infected by HCV, or which are primo-infected by HCV in presence of said antibody. The monitored antibody should be capable of respectively inducing a diminishing of HCV related viremia or to prevent infection by HCV.

The present invention relates to a conformational antibody as defined above, capable of precipitating the HCV E1E2 complex under its covalent or non covalent forms.

The HCV E1E2 complex may be non covalent, that is to say that E1 and E2 are associated by means of weak bonds, such as hydrogen bonding, ionic bonding, Van Der Waals bonding or hydrophobic bonding, or covalent, that is to say that E1 and E2 are associated by means of covalent bonds, such as disulfure bonds for instance. Covalent and non-covalent forms of the E1E2 complex are in particular described or suggested in Deleersnyder *et al.* (1997).

"Precipitating" means that the antibody may render the HCV E1E2 complex insoluble. Precipitation may occur for instance such as described by Dubuisson and Rice (1996).

The present invention relates to a conformational antibody as defined above, capable of specifically binding to the natural HCV E1 protein.

HCV E1 is in particular described in Dubuisson (2000) and Op De Beeck *et al.* (2001). It is produced as a poly-protein (3012 amino acids) which is cleaved to yield E1 (amino acids 192 to 383).

The expression "specifically binding" means that the antibody binds to an epitope, or a part thereof, which is found on substantially only the HCV E1 protein. In particular the antibody can not be detached from the HCV E1 protein in competition tests, wherein the antibody-E1 complex is presented with other proteins.

The expression "natural HCV E1 protein" means that the protein is as found in biological samples of patients infected by HCV, in particular the natural HCV E1 protein is not a recombinant protein.

The present invention more specifically relates to a conformational antibody, capable of specifically binding to the natural HCV E1 protein, to the natural HCV E2 protein, and of precipitating the HCV E1E2 complex under its covalent or non covalent forms.

The present invention relates to a conformational antibody as defined above, capable of specifically binding to an epitope constituted of at least one of the following sequences:

amino acids 297 to 306 of HCV protein E1;

amino acids 480 to 494 of HCV protein E2;

amino acids 613 to 621 of HCV protein E2.

The antibody according to the invention is able to bind to:

- a molecule presenting a peptide comprising amino acids 297 to 306 of HCV protein E1, corresponding to the following sequence: RHWTTQGCNC; and/or
- to a molecule presenting a peptide comprising amino acids 480 to 494 of HCV protein E2, corresponding to the following sequence: PDQRPYCWHYPPKPC; and/or
- to a molecule presenting a peptide comprising amino acids 613 to 621 of HCV protein E2, corresponding to the following sequence: YRLWHYPCT.

The binding of said antibody to at least one of the preceding sequences can be tested by synthesising a peptide containing any of the preceding sequences and by assaying the antibody binding capability to said synthesised peptide by methods well known to the man skilled in the art, such as ELISA or EIA for example.

The acronym "ELISA" means Enzyme Linked Immuno Sorbent Assay.

The acronym "EIA" means Enzyme Immunoassay.

The present invention relates to a conformational antibody as defined above, capable of specifically binding to an epitope constituted of each of the following sequences:

amino acids 297 to 306 of HCV protein E1;

amino acids 480 to 494 of HCV protein E2;

amino acids 613 to 621 of HCV protein E2.

The antibody according to the invention is able to bind to a molecule presenting an epitope, said epitope comprising;

- a peptide comprising amino acids 297 to 306 of HCV protein E1, corresponding to the following sequence: RHWTTQGCNC; and
- a peptide comprising amino acids 480 to 494 of HCV protein E2, corresponding to the following sequence: PDQRPYCWHYPPKPC; and
- a peptide comprising amino acids 613 to 621 of HCV protein E2, corresponding to the following sequence: YRLWHYPCT.

The binding of said antibody to an epitope comprising each of the preceding sequences can be tested by assaying the antibody binding capability to a molecule, in particular a protein, which comprises the sequence RHWTTQGCNC, and to a molecule, in particular a protein, which comprises the sequence PDQRPYCWHYPPKPC, and to a molecule, in particular a protein, which comprises the sequence YRLWHYPCT, by methods well known to the man skilled in the art, such as ELISA or EIA for example.

The present invention relates to a conformational antibody as defined above, wherein said antibody is a monoclonal antibody.

The expression "monoclonal antibody" means that the antibody binds to substantially only one epitope, or to parts of said epitope.

A monoclonal antibody can be obtained from monoclonal cell lines, derived from immortalized antibody secreting cells, such as hybridomas for instance.

5 Monoclonal cell lines are derived from the culturing of a unique cell.

A hybridoma can be produced by fusion of an antibody secreting cell, such as a B cell, with an immortalized cell, according to Kohler and Milstein (1975) or to Buttin *et al.* (1978) for example.

10 According to another aspect of the invention, a monoclonal antibody as defined above is secreted by the hybridoma deposited under the Budapest Treaty at the CNCM (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France) on March 19, 2003, under accession number CNCM I-2983.

15 Said monoclonal antibody in particular binds to the natural HCV viral envelope, to the natural HCV E2 protein, to the natural HCV E1 protein, and is capable of precipitating the HCV E1E2 complex under its covalent or non covalent forms, and is capable of binding to an epitope constituted of at least one or of all of the above-defined sequences. Said monoclonal antibody is in particular referred to hereafter as D32.10.

20 According to another aspect of the invention, a monoclonal antibody as defined above is secreted by the hybridoma deposited under the Budapest Treaty at the CNCM (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France) on March 19, 2003, under accession number CNCM I-2982.

Said monoclonal antibody in particular binds to the natural HCV viral envelope and to the HCV E2 protein. Said monoclonal antibody is in particular referred to hereafter as D4.12.9.

25 The invention also relates to fragments or derivatives of the antibodies according to the invention. Fragments of the antibodies of the invention in particular encompass Fab, F(ab')₂ or scFv (single-chain Fv) fragments, which are well known to the man skilled in the art. Derivatives of the antibodies of the invention comprise, if appropriate, humanized antibodies. Humanized antibodies can be for example chimeric antibodies, in which, if appropriate, parts of the antibodies according to the invention are replaced by the corresponding parts of human antibodies, such as the Fc fragment for instance. Alternatively, the complex determining region (CDR) of the antibodies according to the invention, can be grafted to human antibodies, such as described for instance in US patent number 5,824,307.

30

According to another embodiment, the invention relates to a hybridoma deposited under the Budapest Treaty at the CNCM (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France) on March 19, 2003, under accession number CNCM I-2983.

According to another embodiment, the invention relates to a hybridoma deposited under the Budapest Treaty at the CNCM (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France) on March 19, 2003, under accession number CNCM I-2982.

According to another embodiment, the invention relates to a pharmaceutical composition comprising as active substance at least one of the antibodies as defined above and a pharmaceutically acceptable vehicle.

10 A pharmaceutically acceptable vehicle is in particular as defined in Remington's Pharmaceutical Sciences 16th ed. / Mack Publishing Co.

Other compounds, in particular antiviral compounds can be included in the above defined composition, such as other anti-HCV antibodies and fragments or derivatives thereof, interferons, RNA polymerase inhibitors, protease inhibitors or helicase inhibitors.

15 The above mentioned pharmaceutical composition can be administered in a single dose or multiple doses. In case of a single dose, the composition may comprise from about 0.1 mg of antibody per kg of body weight, to about 1 g of antibody per kg of body weight, in particular from about 1 mg/kg to about 100 mg/kg. In case of multiple doses, the composition can be administered in a dosage of about 0.1 mg of antibody per kg of body weight per day, to about 20 1 g of antibody per kg of body weight per day, in particular from about 1 mg/kg/day to about 100 mg/kg/day.

According to another embodiment, the invention relates to the use of at least one of the antibodies as defined above, for the preparation of a medicament for the diagnostic, the prevention or the treatment of HCV infections.

25 The antibodies according to the invention can be used for the detection of HCV viral particles in biological samples liable to contain HCV. Such samples may be obtained from patients infected by HCV or at risk of being infected by HCV and comprises in particular samples of blood, plasma or serum. Any of the methods well known of the man skilled in the art for the detection of viruses with antibodies, such as ELISA or EIA, can be applied with the 30 antibodies of the invention.

The antibody according to the invention can be used for the preparation of a medicament administered to patients infected or not with HCV, to neutralize HCV infection. HCV infection neutralization can proceed by preventing HCV from contacting target cells, for

instance by binding the antibody to envelope molecules, such as E1 or E2, which bind to target cells membrane receptors, thereby preventing the HCV-target cells binding.

The antibody can be administered to patients infected by HCV to prevent HCV viral particles from infecting cells, in particular hepatocytes, or to promote capture of HCV coated
5 with said antibodies by cells of the immune system.

The antibody can in particular be administered to patients which are to receive a transplanted organ, in particular a liver, prior, during or after the transplantation surgery, to neutralize HCV viral particles which may be contained in the transplanted organ.

The invention also relates to an enveloped viral particle capable of binding to at least one
10 of the antibodies as defined above.

The expression "enveloped viral particle" represents in particular a HCV virion, or part of a HCV virion, which contains an envelope, the envelope comprising lipids and/or proteins, in particular glycoproteins, associated in leaflets.

The invention also relates to an antibody which binds to an enveloped viral particle
15 capable of binding to at least one of the antibodies as defined above.

According to another embodiment, the invention relates to a composition of HCV viral particles derived from initial samples of human blood, plasma or sera, wherein the concentration of HCV RNA copies is about 100 to 1000 fold higher than the concentration of HCV RNA copies in the initial samples of human, blood, plasma or sera from which it is
20 derived, and is in particular higher than about 10^7 copies/ml.

The high concentration of the compositions according to the invention allows efficient immunization of animals with said compositions.

HCV RNA is in particular HCV genomic RNA.

The HCV RNA contents of a sample can be measured by RT-PCR, in particular
25 quantitative RT-PCR, with the AmplicorTM HCV MonitorTM, Roche Diagnostics (Young *et al.*, 1993) for example.

Alternatively, the HCV RNA contents of a sample can also be measured using the NASBA (Nucleic Acid Sequence Based Amplification) technology (Damen *et al.*, 1999).

The HCV RNA contents of a sample can be expressed in terms of number of copies of
30 HCV RNA molecules in said sample, one copy being equivalent to one International Unit (UI).

The HCV RNA contents of a sample is indicative of the quantity HCV virions contained in said sample.

A composition containing more than about 10^7 copies/ml of HCV RNA allows efficient immunization of animals with said composition.

The invention more specifically relates to a composition as defined above, wherein the number of HCV RNA copies is from about 10^8 to about 10^9 UI per mg of protein.

5 The protein content of the composition is assessed by methods well known to the man skilled in the art, and particularly by the Lowry assay (Lowry *et al.*, 1951) such as the Biorad protein assay (Biorad Laboratories).

10 This measure represents the specific activity of the composition, it is indicative of the purity of the composition; the higher the number of HCV RNA copies per mg of protein the higher the purity of the HCV containing composition.

The invention further relates to a composition as defined above, wherein the volume of said composition is from about 0.1 ml to about 10 ml.

A composition volume of about 0.1 ml to about 10 ml corresponds to a composition as defined above containing at least 10^6 HCV RNA copies.

15 Such a composition is useful for the immunization of animals. For example, the efficient immunization of a mouse requires the administration of a HCV viral particles composition containing more than about 10^6 HCV RNA copies, as described in the examples.

According to another embodiment the invention relates to an isolated HCV enveloped subviral particle substantially devoid of HCV RNA and of HCV core protein.

20 The term "isolated" means that the particle has been extracted from its natural environment, it has in particular been separated from other HCV viral particles or parts thereof which contain HCV RNA and /or HCV core protein.

25 The expression "substantially devoid of HCV RNA and of HCV core protein" means that the solution containing the above defined subviral particle contains less than 10^4 UI/ml of HCV RNA as measured with AmplicorTM HCV MonitorTM, Roche Diagnostics (Young *et al.*, 1993), and less than 1 pg/ml of core protein as measured with the Ortho-Clinical Diagnostics test (Aoyagi *et al.*, 1999).

30 The presence of the envelope can be assessed for instance by an EIA or an ELISA test with the antibody secreted by the hybridoma deposited at the CNCM under accession number I-2983, hereafter named D32.10, or the antibody secreted by the hybridoma deposited at the CNCM under accession number I-2982, hereafter named D4.12.9.

The expression "HCV enveloped subviral particle" means that the particle contains substantially only the envelope part of the HCV virion, that is to say lipids and proteins, in

particular glycoproteins, associated in leaflets. HCV viral particles isolated thus far either contained HCV RNA and/or HCV core protein.

The invention more specifically relates to an isolated HCV enveloped subviral particle as defined above, wherein said subviral particle is liable to bind to any of the antibodies as
5 defined above.

The binding of the above defined subviral particle to the antibodies of the invention can be assessed by several methods well known to the man skilled in the art, such as immunoprecipitation, ELISA, EIA or Western blotting for example.

According to another embodiment, the invention relates to a composition comprising
10 purified HCV enveloped complete viral particles, said purified HCV enveloped complete viral particles containing HCV RNA, HCV core protein and HCV envelope, and being liable to bind to any of the antibodies as defined above.

The invention more particularly relates to a composition comprising purified natural HCV enveloped complete viral particles.

15 The expression "HCV enveloped complete viral particles" means that HCV virions contain HCV genomic RNA, HCV core protein and HCV envelope. There has been no report in the prior art of a HCV viral particle containing these three components.

The presence of HCV RNA, HCV core protein and HCV envelope can be assessed by using the methods as defined above.

20 The term "purified" means that the above defined HCV enveloped complete viral particle has been separated from other compounds, such as HCV enveloped subviral particles. In particular, it can be assessed, for example by electron microscopy, that the composition contains 90% less HCV enveloped subviral particles than HCV enveloped complete viral particles.

25 The binding of the above defined HCV enveloped complete viral particle to the antibodies according to the invention can be assessed by several methods well known to the man skilled in the art, such as immunoprecipitation, ELISA, EIA or Western blotting for example.

According to another embodiment, the invention relates to a process for preparing a
30 composition of HCV viral particles comprising the following steps:

at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;

resuspension of the HCV enriched pellet in an aqueous solution to obtain a composition of HCV viral particles.

The ultracentrifugations may be carried out for instance at a speed of about 190,000 g to about 220,000 g, preferably 210,000 g, during about 3 hours to about 5 hours, preferably 4 hours.

5 The preferred centrifugation conditions lead to precipitation of viral particles, such as HCV viral particles, while other compounds contained in the clarified plasma are not precipitated.

The term "plasmapheresis" means that the patient's blood has been filtered to obtain plasma while the remainder of the blood has been re-injected to the patient.

10 The term "clarified" means that the plasma obtained from the plasmapheresis has been centrifuged at low speed, in particular at 3000 g during 30 minutes.

The initial volume of plasmapheresis before ultracentrifugation is advantageously of about 1 litre.

15 According to another embodiment, the invention relates to a composition of HCV viral particles such as obtained according to the above-mentioned process for preparing a composition of HCV viral particles.

According to another embodiment, the invention relates to a process for preparing a composition of HCV enveloped subviral particles comprising the following steps:

- at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;
- 20 - resuspension of the HCV enriched pellet in an aqueous solution;
- ultracentrifugation of the resuspended HCV enriched pellet in a sucrose density gradient to separate the elements of the resuspended HCV enriched pellet into fractions according to their density;
- 25 - recovery of the fractions containing substantially no HCV RNA, substantially no HCV core protein and containing particles capable of binding to the monoclonal antibody defined above as D32.10 or D4.12.9, to obtain a composition of HCV enveloped subviral particles.

30 The ultracentrifugation of the resuspended HCV enriched pellet in a sucrose density gradient maybe carried out from about 190,000 g to about 220,000 g, preferably 210,000 g, during about 40 hours to about 50 hours, preferably 48 hours, the sucrose gradient can be advantageously from about 10% to about 60% w/w, from about 20 to about 50% w/w, or from about 25% to about 45% w/w.

All the fractions obtained are tested for the presence of HCV RNA, HCV core protein and/or particles capable of binding to D32.10 or D4.12.9.

The fractions are said to contain substantially no HCV RNA when the content of HCV RNA as measured by Amplicor™ HCV Monitor™, Roche Diagnostics (Young *et al.*, 1993), is less than about 10^4 UI/ml.

5 The fractions are said to contain substantially no HCV core protein when the content of HCV core protein as measured by the Ortho-Clinical Diagnostics test (Aoyagi *et al.*, 1999) is less than about 1 pg/ml.

The monoclonal antibody used is in particular the monoclonal antibody D32.10 which is secreted by the hybridoma deposited at the CNCM under accession number I-2983

10 The particles are said capable of binding to the above mentioned monoclonal antibody if they can be tested positive in the following EIA test:

Polystyrene plates of 96-wells (Falcon; Becton Dickinson France S.A, Le Pont de Claix) are coated with the different fractions diluted from 10^{-1} to 10^{-4} . The plates are incubated overnight at 4°C and are then saturated with Tris-NaCl (TN) buffer (20 mM Tris-HCl, pH 7.5 and 100 mM NaCl) containing 5% (w/v) bovine serum albumin (BSA). mAb D32.10 diluted
15 in a mixture of TN/BSA buffer and 50% normal human serum (NHS) at a concentration of 5 µg/ml is added to each well and incubated for 2 h at 37°C. The bound antibody is detected with a horseradish peroxidase (HRPO)-conjugated F(ab')₂ fragment of anti-mouse immunoglobulins (diluted 1/5,000; Immunotech) and with orthophenylenediamine (OPD) and hydrogen peroxide (H₂O₂) as substrates. Optical density (OD) is determined at 450 nm or at
20 490 nm with an ELISA plate reader (MRX, Dynex). The results are considered as positive when superior to cut-off, corresponding to mean of negative controls multiplied by 2.1.

The recovered fractions have in particular a sucrose density of approximately 1.13 to 1.15 g/ml.

25 Alternatively, the fractions are first tested for the presence of particles capable of binding to D32.10 and/or D4.12.9, if substantially no such particles are present, then no other test is performed, if such particles are present, then the HCV RNA test and/or the HCV core protein test are performed.

30 According to another embodiment, the invention relates to a composition of HCV enveloped subviral particles such as obtained according to the above process for preparing a composition of HCV enveloped subviral particles.

According to another embodiment, the invention relates to a process for preparing a composition of purified HCV enveloped complete viral particles comprising the following steps:

at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;

resuspension of the HCV enriched pellet in an aqueous solution;

5 ultracentrifugation of the resuspended HCV enriched pellet in a sucrose density gradient to separate the elements of the resuspended HCV enriched pellet into fractions according to their density;

recovery of the fractions containing from about $5 \cdot 10^5$ to about 10^6 UI of HCV RNA per ml, from about 50 to about 100 pg of HCV core protein per ml, and containing particles capable of binding to the monoclonal antibody defined above as D32.10 or D4.12.9, to obtain
10 a composition of purified HCV enveloped complete viral particles.

The ultracentrifugation of the resuspended HCV enriched pellet in a sucrose density gradient maybe carried out from about 190,000 g to about 220,000 g, preferably 210,000 g, during about 40 hours to about 50 hours, preferably 48 hours, the sucrose gradient can be advantageously from about 10% to about 60% w/w, from about 20 to about 50% w/w, or from
15 about 25% to about 45% w/w.

HCV RNA content, HCV core protein content and the binding capability of the particles are measured as indicated above.

The monoclonal antibody used is in particular the monoclonal antibody D32.10 which is secreted by the hybridoma deposited at the CNCM under accession number I-2983.

20 The composition obtained by this process contains in particular purified HCV enveloped complete viral particles and is in particular substantially devoid of HCV enveloped subviral particles. In particular, it can be assessed, for example by electron microscopy, that the composition contains 90% less HCV enveloped subviral particles than HCV enveloped complete viral particles.

25 The recovered fractions have in particular a sucrose density of approximately 1.19 to 1.21 g/ml.

Alternatively, the fractions are first tested for the presence of HCV RNA, if more than 10^5 copies/ml of HCV RNA are present, then the HCV core protein test is performed, if more than 50 pg/ml of core protein are present, then the presence of particles capable of binding to D32.10 and/or to D4.12.9 is tested; if less than 10^5 copies/ml of HCV RNA are present then
30 no other test is performed.

According to another embodiment, the invention relates to a composition of purified HCV enveloped complete viral particles such as obtained according to the above corresponding process.

The invention also relates to a process for preparing a monoclonal antibody secreted by the hybridoma deposited at the CNCM under accession number I-2983, comprising the following steps:

- immunizing an animal, in particular a mammal, with a composition of HCV viral particles of the invention, or such as prepared according to the invention, and recovering the generated antibodies;
- selecting, among the generated antibodies, monoclonal antibodies on their ability of binding to the HCV viral particles contained in the above mentioned composition of HCV viral particles.

The composition of HCV viral particles can be obtained as follows:

- at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;
- resuspension of the HCV enriched pellet in an aqueous solution to obtain a composition of HCV viral particles.

The selection of the generated antibodies can be carried out with an indirect EIA test as defined above, the plates being coated with the composition of HCV viral particles of the invention.

According to another embodiment, the invention relates to a process for preparing a monoclonal antibody secreted by the hybridoma deposited at the CNCM under accession number I-2982, comprising the following steps:

- immunizing an animal, in particular a mammal, with a composition of purified HCV enveloped complete viral particles of the invention, or such as prepared according to the invention, and recovering the generated antibodies;
- selecting, among the generated antibodies, monoclonal antibodies on their ability of binding to the purified HCV enveloped complete viral particles contained in the above mentioned composition of purified HCV enveloped complete viral particles.

The composition of purified HCV enveloped complete viral particles can be obtained as follows:

- at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;
- resuspension of the HCV enriched pellet in an aqueous solution;

- ultracentrifugation of the resuspended HCV enriched pellet in a sucrose density gradient to separate the elements of the resuspended HCV enriched pellet into fractions according to their density;
- recovery of the fractions containing from about $5 \cdot 10^5$ to about 10^6 UI of HCV RNA per ml, from about 50 to about 100 pg of HCV core protein per ml, and containing particles capable of binding to the monoclonal antibody defined above as D32.10 or D4.12.9, to obtain a composition of purified HCV enveloped complete viral particles.

The selection step can proceed as described above.

According to another embodiment, the invention relates to a process for preparing a monoclonal antibody directed against HCV, in particular HCV enveloped subviral particles, comprising the following steps:

- immunizing an animal, in particular a mammal, with a composition of HCV enveloped subviral particles of the invention, or prepared according to the invention, and recovering the generated antibodies;
- selecting, among the generated antibodies, monoclonal antibodies on their ability of binding to the HCV enveloped subviral particles contained in the above mentioned composition of HCV enveloped subviral particles.

The composition of HCV enveloped subviral particles can be obtained as follows:

- at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;
- resuspension of the HCV enriched pellet in an aqueous solution;
- ultracentrifugation of the resuspended HCV enriched pellet in a sucrose density gradient to separate the elements of the resuspended HCV enriched pellet into fractions according to their density;
- recovery of the fractions containing substantially no HCV RNA, substantially no HCV core protein and containing particles capable of binding to the monoclonal antibody defined above as D32.10 or D4.12.9, to obtain a composition of HCV enveloped subviral particles.

The selection step can proceed as described above.

The present invention more particularly relates to antibodies directed against the HCV enveloped subviral particles of the invention.

The invention also relates to a pharmaceutical composition comprising as active substance at least one antibody directed against HCV enveloped subviral particles and a pharmaceutically acceptable vehicle.

5 According to another embodiment, the invention relates to a pharmaceutical composition comprising as active substance the HCV enveloped subviral particles as defined above, or the composition comprising the HCV enveloped subviral particles as defined above, and a pharmaceutically acceptable vehicle.

10 Additionally adjuvants, such as defined for instance in Remington's Pharmaceutical Sciences 16th ed. / Mack Publishing Co. can be added to the pharmaceutical composition, adjuvants may be for instance incomplete Freund's adjuvant, aluminum salts or aluminum hydroxide.

The composition can be administered, for instance, in a single dose comprising from 1 mg to 1 g of HCV enveloped subviral particles.

15 According to another embodiment, the invention relates to the use of the HCV enveloped subviral particles as defined above, or of the composition comprising the HCV enveloped subviral particles as defined above, to induce an immune reaction against said HCV enveloped subviral particles or against HCV enveloped complete viral particles as defined above.

20 The expression "induce an immune reaction" means that B cells secreting antibodies directed against HCV viral particles can be activated or that T cells destroying cells infected by HCV can be activated.

25 According to another embodiment, the invention relates to the use of the HCV enveloped subviral particles as defined above, or of the composition comprising HCV enveloped subviral particles defined above, for the preparation of a medicament for the diagnostic, the prevention or the treatment of HCV infections.

The HCV enveloped subviral particles can be used to assess the presence of antibodies directed against HCV in immunoassays according to method well known to the man skilled in the art, such as EIA, ELISA.

30 The HCV enveloped subviral particles can be used for the preparation of a vaccine against hepatitis C, in particular a therapeutic vaccine.

The expression "therapeutic vaccine" means that the vaccine is capable of improving the condition of patient infected by HCV, for example by inducing the production of antibodies directed against HCV.

Brief description of the drawings

Figure 1A, Figure 1B, Figure 1C, Figure 1D and Figure 1E

Figure 1A, 1B, 1C, 1D and 1E represent sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) of antibody precipitated lysates of ^{35}S labelled cells. Lanes 1, 2, 3, 4, 5, 6 and 7 correspond respectively to antibodies C9.19.16, C2.22.1, D32.10, D3.20.12, C7.24.19, C7.14.41 and C1.9.3. MW corresponds to a molecular weight marker. The size of the bands corresponding to the molecular weight marker, are presented on the right of the gel. When possible the bands are identified on the left of the gel as E1, E2 or Agg (for aggregated).

Figure 1A represents a SDS-PAGE of antibody precipitated lysates of ^{35}S labelled control cells, in reducing conditions.

Figure 1B represents a SDS-PAGE of antibody precipitated lysates of ^{35}S labelled cells expressing HCV E1 protein, in reducing conditions.

Figure 1C represents a SDS-PAGE of antibody precipitated lysates of ^{35}S labelled cells expressing HCV E2 protein, in reducing conditions.

Figure 1D represents a SDS-PAGE of antibody precipitated lysates of ^{35}S labelled cells expressing HCV E1E2 protein, in reducing conditions.

Figure 1E represents a SDS-PAGE of antibody precipitated lysates of ^{35}S labelled cells expressing HCV E1E2 protein, in non-reducing conditions

Figure 2

Figure 2 represents the Western blotting of HCV viral particles, using D32.10 monoclonal antibody, under non-reducing and reducing conditions. From left to right, M represents a molecular weight marker, under non-reducing conditions, the size of its bands being indicated on the left of the gel (in kDa), lane 1 represents the Western blotting of HCV viral particles, using D32.10 monoclonal antibody, under non-reducing conditions, second lane M represents a molecular weight marker, under reducing conditions, lane 2 represents the Western blotting of 2,5 μg of HCV viral particles, using D32.10 monoclonal antibody, under reducing conditions, lane 3 represents the Western blotting of 5 μg of HCV viral particles, using D32.10 monoclonal antibody, under reducing conditions. On the right of the gel, the size of several bands of lane 3 are indicated (in kDa), some corresponding to HCV protein E2 (60 and 68) or to HCV protein E1 (34 and 31).

Figure 3A and Figure 3B

Figure 3A and 3B represent the Western blotting of HCV viral particles submitted to deglycosylation by glycosidase A (Figure 3A) and by endoglycosidase H (Figure 3B).

- 5 In figure 3A, lane M represents a molecular weight marker, the size of three of its bands being indicated (in kDa) on the left of the gel; lane 1, 2, 3 and 4 represent respectively glycosidase concentrations of 20, 10, 5 and 0 mU/ml. On the right of the gel, the positions of HCV proteins E2 and E1 are indicated, as well as the position of a major deglycosylated form of protein E1 (E1*) and the size (in kDa) of several bands corresponding to deglycosylated forms of E1 or E2, as well as the major deglycosylated form of E2 (41*).
- 10 In figure 3B, lane M represents a molecular weight marker, the size of four of its bands being indicated (in kDa) on the left of the gel; lane 1 and 2 represent respectively a control deglycosylation experiment carried out without endoglycosidase H and a deglycosylation experiment carried out in presence of endoglycosidase H. On the right of the gel are represented the major bands corresponding to the fully glycosylated forms of E2 and E1 and to deglycosylated forms of E2 (50, 48, 46 and 42 kDa) and E1 (28 and 24 kDa). The predominant deglycosylated forms of E2 (50*) and E1 (28*) are marked by a star.
- 15

Figure 4

- 20 Figure 4 represents the characterization of the fractions obtained by centrifugation on a sucrose density gradient of a HCV viral particles preparation. Three parameters have been measured, HCV RNA content, HCV core protein content and reactivity towards D32.10 monoclonal antibody. The horizontal axis represents the numbers of the fractions submitted to characterization. The left vertical axis represents HCV RNA concentration ($\times 10^5$ UI/ml) and a measure of the reactivity towards D32.10 as measured by indirect ELA (OD at 450 nm). The
- 25 right vertical axis represents the HCV core protein concentration (in pg/ml). The curve corresponding to the black dots represents the reactivity of the fractions towards D32.10, the curve corresponding to the white triangles represents the fractions contents in core protein and the dotted bars represent the fractions contents in HCV RNA.

Figure 5A, Figure 5B and Figure 5C

Figure 5A, 5B and 5C represent respectively transmission electron microscopy pictures of a HCV viral particles preparation, of a HCV enveloped complete viral particles preparation and of a HCV enveloped subviral particles preparation.

In figure 5A, the horizontal bar represents a scale of 200 nm in length. The larger circular elements represent HCV enveloped complete viral particles and the smaller circular elements represent HCV enveloped subviral particles.

In figure 5B, the horizontal bar represents a scale of 50 nm in length. The circular elements represent HCV enveloped complete viral particles.

In figure 5C, the horizontal bar represents a scale of 50 nm in length. The circular elements represent HCV enveloped subviral particles.

Figure 6

Figure 6 represents the Western blotting of HCV viral particles, using D4.12.9 monoclonal antibody. Lanes M represent a molecular weight marker, the size of four of its bands (75, 50, 37, 25) being indicated (in kDa) on the left side and on the right side of the gel. Lanes 1 and 2 represent the result of a digestion of the HCV viral particles by glycosidase A and by endoglycosidase H respectively, lanes 3 and 4 represent the result of a proteolytic digestion of HCV viral particles by proteases trypsin and V8, lane 5 represents the result of lysis by NP-40, and in lane 6 no prior treatment as been performed. On the right of the gel, the two major forms of undigested E2 protein (68 and 48) are indicated (in kDa), and on the left of the gel the major deglycosylated form of E2 (E2*) is represented.

Figure 7

Figure 7 represents a D4.12.9 based characterization of fractions obtained by centrifugation on a sucrose density gradient of a HCV viral particles preparation. In addition to the fractions reactivity towards D4.12.9, the fractions contents in core protein was also measured. The horizontal axis represents the numbers of the fractions submitted to characterization. The right vertical axis represents a measure of the reactivity towards D4.12.9 as measured by indirect EIA (OD at 450 nm). The left vertical axis represents the HCV core protein concentration (in pg/ml). The curve corresponding to the black squares represents the reactivity of the fractions towards D4.12.9, the curve corresponding to the black diamond shapes represents the fractions contents in core protein.

Figure 8A, Figure 8B and Figure 8C

Figure 8A, 8B and 8C represent the immunoreactivity of three biotinylated peptides, comprising respectively amino acids 290-317 (Figure 8A), amino acids 471-500 (Figure 8B) and amino acids 605-629 (Figure 8C), towards 55 sera of healthy individuals (11 sera,

numbered T1 to T11 on the horizontal axis) and HCV infected patients (44 sera, numbered A1 to A44 on the horizontal axis). The vertical axis represents the reactivity of the sera to the three peptides as measured by ELISA (OD at 492 nm x1000). The white bars represent the reactivity of the healthy individuals' sera and the grey bars represent the reactivity of the infected patients' sera. The horizontal line represents a cut-off value above which a serum response is considered positive. In figure 8A, the cut-off value is 0.691, in figure 8B, 0.572 and in figure 8C, 0.321.

EXAMPLE 1**Obtaining a monoclonal antibody directed against the natural HCV viral envelope****HCV viral particles preparation**

To obtain virus materials in good supply, the purification of HCV viral particles was performed from plasmaphereses. The selected patient developed chronic active hepatitis C after partial liver transplantation for C viral cirrhosis, and 12-months later multiple myeloma associated with hypergammaglobulinemia which was the cause of plasma exchange. The patient showed abnormal elevated serum aminotransferase (ALT/AST) levels and was positive for anti-HCV antibodies. The patient was negative for all HBV and HIV markers. The HCV RNA content of initial material was 6×10^5 copies/ml (AmplicorTM HCV MonitorTM, Roche Diagnostics, Meylan, France), and the genotype 1b (INNO-LIPA assay, Innogenetics, Gent, Belgium).

Clarified plasmapheresis was used to prepare a HCV-enriched pellet by two successive ultracentrifugations at 210,000 g for 4 h at 4°C. The final pellet was resuspended in 1 ml of Tris-NaCl-EDTA (TNE) buffer (20 mmol/L Tris-HCl, pH 7,5, 100 mmol/L NaCl, 1 mmol/L EDTA) (concentration 240 fold), and stored at -80°C. The HCV RNA content of this material was 3×10^7 copies/ml (Monitor, Roche) and the protein concentration 5 mg/ml (i.e. about 10^7 copies of HCV RNA per mg of protein).

Hybridoma preparation

For generation of monoclonal antibodies (mAbs), BALB/c mice were inoculated with 100 µg (10^6 copies of HCV RNA) of HCV-pelleted material in complete Freund adjuvant, followed 1 week later with 100 µg of virus in incomplete Freund adjuvant. The three immunized mice developed high serum antibody titers against HCV, detected by indirect enzyme immunoassay (EIA). Three weeks later, mice were boosted with 50 µg of virus in phosphate buffered saline (PBS). After 5 days the injection was repeated and 3 days later the spleen was removed for fusion with X63 myeloma cells by the procedure described by Buttin *et al.* (1978). Hybridoma culture supernatants were screened for the presence of HCV-specific antibodies by indirect EIA using the immunogen as the solid phase (5 µg/ml) and peroxidase-conjugated F(ab')₂ fragment of anti-mouse immunoglobulins (Amersham, France) as a revelation secondary antibody (Petit *et al.*, 1987). The diluent contained 50% normal human serum (NHS) to eliminate non-specific reactivity directed against NHS proteins possibly

associated with HCV viral particles. The hybrids giving the strongest signal ($P/N > 10$) to HCV were then recloned by limiting dilution and their specificity further determined. Seven clones (C9.19.16, C2.22.1, D32.10, D3.20.12, C7.24.19, C7.14.41 and C1.9.3) were selected and four (D32.10, C2.22.1, C9.19.16 and D3.20.12) were propagated by injection into
 5 pristane-primed BALB/c mice for ascitic fluid production, and then purified by precipitation with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ followed by affinity chromatography in Sepharose-Protein G (Pharmacia, France). Isotype was determined by ELISA with methods well known to the man skilled in the art. The seven clones analyzed gave antibodies of the IgG1 isotype.

10 Monoclonal antibody characterization

Indirect EIA was used to evaluate the interaction of the above mentioned monoclonal antibodies with the viral particles. Polystyrene plates of 96-wells (Falcon; Becton Dickinson France S.A, Le Pont de Claix) were coated with the HCV preparation (1 mg of protein per ml) diluted from 10^{-1} to 10^{-6} (corresponding to 100 $\mu\text{g/ml}$ to 1 ng/ml). The plates were incubated
 15 overnight at 4°C and were then saturated with Tris-NaCl (TN) buffer (20 mM Tris-HCl, pH 7.5 and 100 mM NaCl) containing 5% (w/v) bovine serum albumin (BSA). mAb D32.10 diluted in a mixture of TN/BSA buffer and 50% normal human serum (NHS) at a concentration of 5 $\mu\text{g/ml}$ was added to each well and incubated for 2 h at 37°C . The bound antibody was detected with the horseradish peroxidase (HRPO)-conjugated F(ab')_2 fragment
 20 of anti-mouse immunoglobulins (diluted 1/5,000; Immunotech) and with orthophenylenediamine (OPD) and hydrogen peroxide (H_2O_2) as substrates. Optical density (OD) was determined at 450 nm with an ELISA plate reader (MRX, Dynex). The results were considered as positive when superior to cut-off, corresponding to mean of negative controls multiplied by 2.1. The seven antibodies obtained did recognize the viral particles.

25 To establish the native polypeptide specificity of the mAbs, immunoblotting using the immunogen as antigenic probes (Petit *et al.*, 1987) was carried out. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 12.5% gels was performed under reducing conditions (2% SDS + 5% 2-ME). After protein transfer onto PVDF membranes, mAbs (2 to 5 $\mu\text{g/ml}$) were tested as primary antibodies, diluted in 50% NHS, and IgGs bound
 30 were detected by incubation with peroxylase-conjugated F(ab')_2 fragment of anti-mouse immunoglobulins (diluted 1/10,000; IMMUNOTECH), as secondary antibody. Bands were visualized by enhanced chemoluminescence (ECL+) system from Amersham.

All mAbs tested, except D32.10, gave negative reactions with HCV polypeptides under reducing conditions. It is worth noting that the mAbs selected were not reactive with either

human serum albumin or with γ - or μ -chains of immunoglobulins (Ig), except C1.9.3 and C7.24.19 which faintly reacted with human IgG in EIA (approximately 5-fold the negative value).

The polypeptide specificity of the seven antibodies was also examined by immunoprecipitation. HepG2 cells infected by vaccinia virus recombinants expressing HCV proteins (E1, E2 or E1E2) were metabolically labeled with ^{35}S -Translabel (ICN) as previously described (Dubuisson *et al.*, 1996). Cells were lysed with 0.5% NP-40 in 10 mM Tris-HCl (pH7.5), 150 mM NaCl, and 2 mM EDTA. The precipitates were treated with Laemmli sample buffer and analyzed by SDS-PAGE under reducing or non-reducing conditions (**Figure 1**). The monoclonal antibodies tested did not show non specific reactivity directed against cellular components, except C9.19.16 (> 200 kDa), D3.20.12 (three intense bands at 70, 50 and 46 kDa) and more faintly, C2.22.1 (70, 50, 46 kDa) and D32.10 (multiple very faint bands) (**Figure 1A**, control vector). All antibodies specifically immunoprecipitated E1 (**Figure 1B**) and E2 (**Figure 1C**), when the two HCV glycoproteins were expressed separately, as well as E1E2 heterodimers (**Figure 1D**), when these proteins were co-expressed. Interestingly, the pattern was different after SDS-PAGE under non-reducing and reducing conditions (**Figure 1D, 1E**). All recognized disulfide-linked E1E2 aggregates, which were detected in the upper part of the gels under non-reducing conditions (**Figure 1E**). One mAb, D32.10, was found to react mostly with aggregates and also with E1E2 non covalently-linked mature complex (**Figure 1E**, non-reducing conditions). All mAbs did not recognize denatured recombinant E1 or E2 proteins expressed in heterologous system by Western blot analysis, suggesting they recognized conformational epitopes on HCV viral particles. Collectively, these results indicate that these mAbs specifically recognize disulfide-linked E1E2 complexes expressed at the surface of natural serum-derived HCV viral particles. It is highly probable that they react with a shared conformational epitope between E1 and E2 proteins. Monoclonal antibody D32.10, which interacts with the E1E2 complex under its covalent or non-covalent form was chosen for further studies.

Monoclonal antibody D32.10 antigen mapping

To test the HCV type specificity of D32.10, an indirect EIA was carried out, according to the procedure already described, with four different HCV preparations (1 mg of protein per ml) diluted from 10^{-1} to 10^{-6} (corresponding to 100 $\mu\text{g/ml}$ to 1 ng/ml). In addition to the immunogen HCV preparation, a preparation obtained from the same patient was used, as well as two other preparation obtained from two different patients with chronic hepatitis C, one of

the patient having been found to carry to distinct genotypes in serum, namely HCV1a and HCV2a. D32.10 was found able to recognize all four of the HCV preparations, thereby indicating that it is able of recognizing determinants not restricted to the 1b genotype of the immunogen.

5 To assess the native polypeptide specificity of D32.10 a Western blot analysis was carried out. Untreated HCV-enriched pellet was used as the antigenic probe, at different concentrations, varying from 0.1 to 1 mg/ml. The antigen was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels under reducing or non-reducing conditions (2% SDS \pm 5% 2-mercaptoethanol (2-ME)). After protein transfer
10 onto PVDF membranes, immunoblotting was performed using mAb D32.10 (2 to 5 μ g/ml) as primary antibody, diluted in 50% NHS. Mouse IgGs bound were then detected by incubation with peroxidase-conjugated (Fab')₂ fragment of anti-mouse immunoglobulins (diluted 1/10,000; DAKO), as secondary antibody. Protein bands were visualized by enhanced chemiluminescence (ECL+) system from Amersham. Glycosidase digestion was performed as
15 previously described by Sato *et al.* (1993) on circulating HCV virions. The HCV-enriched pellet (HCV-L, 4 μ g) was treated with 5, 10 or 20 mU/ml of glycosidase A (peptide-N-glycosidase A or PNGase A; ROCHE) in 100 mM citrate/phosphate buffer (pH 6.0) for 18 h at 37°C. Deglycosylation of purified HCV viral particles was also performed by incubation overnight at 37°C in 50 mM sodium acetate buffer (pH 5.5) containing endoglycosidase H
20 (endo H, 5 mU/ μ l from Roche), 20 mM dithiothreitol (DTT) and 0.1% Triton X100. The control digestion was performed using the same conditions as for the PNGase A digestion or the endo H digestion, except that the enzyme was omitted. Samples were then treated with electrophoresis sample buffer containing reducing agent and analyzed by SDS-PAGE.

The results of the Western blot experiment are presented in Figure 2. When two
25 concentrations of the same sample (2.5 and 5 μ g, lanes 2 and 3, respectively) were analyzed under reducing conditions (2% SDS/5% 2-ME), mAb D32.10 recognized a major band of 68 kDa and another band of 31 kDa, corresponding to E2 and E1 respectively. However, under non-reducing conditions, mAb 32.10 recognized disulfide linked complexes recovered in the upper part of the gel (>200kDa). These high molecular weight bands (Figure 2, lane 1) very
30 probably correspond to heterodimeric E1E2 complexes.

Asparagine-linked complex type sugar chains have been shown to be present on the surface of native virions of HCV (Sato *et al.*, 1993), thus the ability of D32.10 mAb to recognize HCV-specific proteins after treatment of the HCV preparation with glycosidase

(PNGase A) at different concentrations (20, 10 and 5 mU/ml) was examined. As shown in **Figure 3A** (lanes 1, 2 and 3, respectively), D32.10 reacted with deglycosylated forms of E1, especially the 25 kDa species, which accumulated at the highest concentration of the enzyme. While E1-related products could be clearly detected by D32.10 after deglycosylation, the E2-related products were not clearly identified after the treatment. Endoglycosidase H (endo H) digestion allowed the investigation of the sensitivity of E1E2 complexes expressed on natural HCV viral particles to endo H cleavage. As shown in **Figure 3B**, a shift in molecular weight was observed for both E2 (from 68 to 42 kDa) and E1 (from 34 to 24 kDa) proteins suggesting that E1 and E2 possess a complex glycosylation on serum-derived native HCV viral particles, accounting for partial endo H resistance, such as a mixture of endo H resistant complex glycans and sensitive forms.

D32.10 epitope mapping

a. Screening of a peptide library

To further characterize the epitope recognized by mAb D32.10, the antibody was used to screen a dodecapeptide phage display library.

A Ph.D.-12TM Phage Display Peptide Library Kit was obtained from New England BioLabs Inc. This is a combinatorial peptide 12-mers fused to the minor coat protein (pIII) of M13 phage. The displayed peptide 12-mers are expressed at the N-terminus of pIII. The library consists of about 1.9×10^9 electroporated sequences, amplified once to yield about 20 copies of each sequence in 10 μ l of the supplied phage. Three biopannings were performed according to the instruction of the manufacturer with some modifications. Briefly, 10 μ g of biotinylated mAb D32.10 were coupled to 35 mm polystyrene Petri dish (Falcon) coated with 40 μ g of streptavidin. The dish was incubated overnight at 4°C and washed six times with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS) containing 0.5% Tween-20 (TBS-T). In the first round of selection, 4×10^{10} phages from the initial library were allowed to react with the dish bound IgG for 4h at 4°C under rocking condition. The unbound phages were removed by repetitive washes with TBS-T. The bound phages were then eluted from the dish with 400 μ l of elution buffer (0.1 N HCl, pH adjusted to 2.2 with glycine, 1 mg/ml BSA). After neutralisation with 75 μ l of 1 M Tris-HCl pH 9.1, the eluted phages were then amplified by infecting 20 ml of a 1:100 dilution of an overnight culture of *E. coli* ER2537 (recA+ strain cells), as recommended in the instruction manual. The culture was incubated for 4.5 h at 37°C with vigorous shaking. The supernatants were obtained and precipitated with polyethyleneglycol (PEG) as previously described (Scott *et al.*, 1990). In the second and third

rounds of selection, 20% of the amplified phages from the preceding round were preincubated overnight at 4°C with the biotinylated mAb D32.10 at the final concentration of 10 and 1 nM, respectively, before being added to the 35-mm polystyrene Petri dish coated with 10 µg of streptavidin. The procedure was then identical to the first round. The phages from the third
 5 biopanning eluate were then cloned and amplified for DNA sequencing and immunoanalysis.

For DNA sequencing, single-stranded DNA was prepared from the purified phages as described by Sambrook *et al.* (1982). The nucleotide sequence of the gene III inserts was determined according to the modified method of Sanger (Sanger *et al.*, 1977) with an Applied Biosystems DNA sequencer (Model 377A) using the BigDyeTM Terminator Cycle Sequencing
 10 Ready Reaction kit (Perkin-Elmer). Cycle sequencing was performed with a primer 5' HO-CCCTCATAGTTAGCGTAACG-OH 3' corresponding to the pIII gene sequence. The amino acid sequence of the insert was deduced from the nucleotide sequence.

For ELISA on supernatant phages, rows of ELISA plate wells were coated with 100 µl of either mAb D32.10 or an irrelevant mAb at the final concentration of 100 µg/ml in 0.1 M
 15 NaHCO₃ buffer (pH 8.6). The plates were incubated overnight at 4°C and then were blocked with 0.1 M NaHCO₃ buffer (pH 8.6) containing 5 mg/ml of BSA. After 2 h incubation at 4°C the plates were washed six times with TBS containing 0.5% Tween. Four fold serial dilutions of each phage clone were added to each well of the microtiter plate in a final volume of 100
 20 µl of TBS-T, starting with 10¹² virions in the first well of a row and ending with 2x10⁵ virions in the 12th well. The plates were incubated for 2 h at room temperature with agitation and were then washed six times with TBS-T as above. The bound phages were detected in a sandwich assay using a horseradish peroxidase-conjugated anti-M13 mAb at a 1:5,000 dilution (Pharmacia). The plates were developed using a commercial color kit (bioMérieux) containing OPD and H₂O₂. After 10 min of incubation, the plates were read at 492 nm with an
 25 ELISA plate reader. For each phage clone dilution, the results were expressed as the difference between the value obtained with the tested anti-HCV mAb and the value obtained with the irrelevant mAb. The results were then confirmed by testing optimal dilutions of the immunoreactive clones in triplicate.

For sequence analysis, the amino acid sequences of peptides were compared to the HCV
 30 E1 and E2 protein sequences by use of the Mac Vector, Ver. 4.5 software (Kodak). Basically, the regions of highest similarity were detected with the LFASTA program, which tentatively searches for best local identities (Pearson and Lipman, 1988).

After the three rounds of selection, 4% of the phage input were found in the eluate indicating amplification of specifically bound phages. Thus, 88 clones were randomly

isolated, their DNA were sequenced and the amino acid sequences of inserts were deduced. Forty eight different sequences were obtained and some of them were found in several examples. However, when tested in an ELISA test for their immunoreactivity with D32.10, none of them gave a positive signal indicating that the binding affinity was too low to be detectable. The 48 clone sequences were compared to the sequences of HCV E1 and E2. Five and three sequences presented similarities with residues of E1 located in the 292-305 region and in the 347-356 region respectively (similarities are indicated in bold in Table 1), whereas 7, 4 and 2 sequences shared some similarities with residues of E2 located in the regions 481-501, 610-631 and 685-698 respectively (similarities are indicated in bold in Table 2).

E1 (289-307)	QLFTFSRRHWTTTQGCNCS
Clone 1	SPLRHYELPLIQ
Clone 2	WPHNHSTHSRTH
Clone 3	FPKYHPRFHKHA
Clone 4	SQSRHWHDVVK
Clone 5	TSQPRWHQKPAT
E1 (343-363)	AILDMIAGAHWGVLAGIAYFS
Clone 6	WKMPRATDWNLR
Clone 7	HWGNHSHKSHQOR
Clone 8	WHRTPSTLWGI

Table 1

E2 (481-501)	DORPYCWHYPKPCGIVPAKS
Clone 9	WHKLPGHPRTV
Clone 4	SQSRHWHDVVK
Clone 10	TFAWHKPRVNLG
Clone 11	TSQPRWHQKPAT
Clone 12	HSSWYIQHFPPL
Clone 13	FPAHPLPRLPSL
Clone 8	WHRTPSTLWGI
E2 (610-631)	DYPYRLWHYPCTINYTIFKIRM
Clone 1	SPLRHYELPLIQ
Clone 14	WHWNKPIIRPPLR
Clone 15	QPYKLQAAATLY
Clone 6	WKMPRATDWNLR
E2 (685-698)	LSTGLIHLHQNIVD
Clone 16	HLYHKNRNHHIAY
Clone 17	WSPGQORLHNST

Table 2

b. Peptide synthesis

In order to evaluate the significance of these different localizations on both E1 and E2 sequences, the regions 291-315 and 347-356 of E1 as well as the regions 473-498, 607-627 and 686-697 of E2 were reproduced as overlapping synthetic pentadecapeptides offset by one and tested for their immunoreactivity with D32.10.

The simultaneous synthesis of different peptide sequences was performed on a nitrocellulose membrane using 9-fluorenylmethoxycarbonyl aminoacid chemistry (Frank, 1988)

Each peptide was generated in nanomolar quantities suitable for immunological detection. Antibody reactivity to membrane bound peptides was analyzed by an indirect colorimetric immunoassay as described previously (Jolivet-Reynaud, 1998). Spots corresponding to

peptides with antibody reactivity produced a positive blue signal. Intensity of spots was estimated by visualization and expressed as relative intensity on a scale ranging from 0 to 5.

A strong positive signal was obtained with peptides corresponding to the 292-305 E1 region whereas the 347-356 E1 region was not recognized by D32.10. Peptides corresponding to the E2 regions 482-499 and 612-626 respectively were also immunoreactive with D32.10 and no signal was detected with the E2 region 686-697. The regions 292-306 of E1 as well as the regions 480-494, and 608-622 of E2 as pentadecapeptides interacted with D32.10 in ELISA.

By using overlapping octapeptides, the ²⁹⁷RHWTTQGCNC³⁰⁶ region of the HCV E1 protein and both ⁶¹³YRLWHYPCT⁶²¹ and ⁴⁸⁰PDQRPYCWHYPPKPC⁴⁹⁴ regions of the HCV E2 protein were reactive with D32.10. The two regions identified in E2 contain the same motif WHYP suggested by Yagnik *et al.* (2000) to be involved in the heterodimerization of E1E2. Indeed it is difficult to discriminate these regions, but as two non overlapping zones: ⁴⁷⁹GPDQRPYC⁴⁸⁶ and ⁴⁸⁷WHYPPKPC⁴⁹⁴ separately bound to D32.10, this suggests that D32.10 specifically recognizes each octapeptide, and so the complete sequence (480-494).

EXAMPLE 2

Characterization and purification of serum derived HCV viral particles

To separate the different HCV populations, the final HCV-enriched pellet was subjected to isopycnic centrifugation (210,000 g for 48 h at 4°C) in a sucrose density gradient (10 to 60%, w/w). Fractions (0.6 ml) were collected, and the density of each was determined by refractometry. HCV-RNA content was analyzed by quantitative RT-PCR (Amplicor Monitor, Roche), HCV core protein content was measured with the Ortho-Clinical Diagnostics test and the HCV viral particles antigenic reactivity towards mAb D32.10 was measured by indirect EIA.

Indirect EIA was carried out as indicated above except that the wells were coated with the different fractions diluted from 10⁻¹ to 10⁻⁴.

Three different populations were identified (**Figure 4**). One population (fractions 3 to 5) banded at a sucrose density of 1.06-1.08 g/ml and was substantially devoid of viral envelope as evidenced by the negative results obtained by indirect EIA with D32.10, but contained HCV RNA (about 2.10⁵ UI/ml) and HCV core protein (from about 2 to 4 pg/ml). These seem to be non-enveloped viral particles. Another population (fractions 8 to 10 of

figure) banded at a sucrose density of 1.14-1.15 g/ml and was substantially devoid of HCV RNA (less than about 10^4 to 10^5 UI/ml) and of HCV core protein (about 1 pg/ml) but contained high levels of particles responding to D32.10 (from about 1 to 3.8 OD_{450 nm} units). These HCV subviral particles seem to contain only the HCV viral envelope. The third population (fractions 11 to 14) banded at a sucrose density of 1.20-1.21 g/ml and contained particles with high levels of HCV RNA (more than about $5 \cdot 10^5$ to 10^6 UI/ml) and of HCV core protein (from about 2.5 to 8 pg/ml) and responding to D32.10 (from about 0.5 to 1.5 OD_{450 nm} units). Hence, this population contains substantially only purified HCV enveloped complete viral particles.

The viral particles contained in the HCV-enriched pellet (**Figure 5A**), and in the second (**Figure 5C**) and third populations (**Figure 5B**) were immunoprecipitated by D32.10 and observed by electron microscopy. Several preparations of HCV viral particles have been analysed by this procedure. The HCV enveloped subviral particles appear as spherical particles with an average diameter of about 30 nm (33.08 nm) (**Figure 5C**), whereas the HCV enveloped complete viral particles appear equally spherical but with an average diameter of about 50 nm (48.72 nm) (**Figure 5B**).

EXAMPLE 3

Obtaining of a monoclonal antibody directed against purified HCV enveloped complete viral particles

The fraction of the above mentioned HCV-enriched pellet subjected to isopycnic centrifugation in a sucrose density gradient, containing the purified HCV enveloped complete viral particles, was used to prepare a monoclonal antibody. This fraction was used to immunize mice and isolate hybridomas as explained above. Thus, one monoclonal antibody, (D4.12.9) was obtained, which was shown to specifically recognize the natural HCV E2 protein in Western blotting experiments (**Figure 6**) and the natural HCV viral particles evidenced in Example 2 (**Figure 7**). Indeed, **Figure 7** shows that D4.12.9 recognizes both the HCV enveloped subviral particles (fractions 8 to 11) and the HCV enveloped complete viral particles (fractions 11 to 14), in a manner similar to D32.10.

EXAMPLE 4**Epitopic characterization of anti-HCV antibodies derived from infected patients**

5 The immunoreactivity of the E1 and E2 derived peptidic sequences reactive with D32.10 (Example 1) towards sera of HCV-infected patients was assessed by using peptides encompassing them.

10 E1 (amino acids 290-317) and E2 (amino acids 471-500 and 605-629) sequences were produced as biotinylated synthetic peptides to be tested by ELISA with 11 sera from healthy individuals and 44 sera from HCV-infected patients (numbered A1 to A44).

15 The wells of microtitration plates were coated overnight at 4°C with 100 µl of streptavidin at the concentration of 10 µg/ml in 0.1 M carbonate buffer (pH 9.6) and blocked for 1 h at 37°C with PBS containing 10% goat serum. The plates were then washed three times with PBS containing 0,05% Tween -20 before adding 100 µl of a biotinylated peptide solution (10 µg/ml in PBS) for 2 h at 37°C. After a new wash with PBS-Tween, 100 µl of the tested serum diluted 1:50 in PBS-Tween containing 10% goat serum was added and incubated for 2 h at 37°C. The plates were washed again with PBS-Tween. The secondary antibody, peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories), was then added at a 1:5000 dilution in PBS-Tween-goat serum. The plates were incubated 1 h at 20 37°C and then washed once more with PBS-Tween. The plates were developed using the Biomerieux color kit containing o-phenylenediamine and hydrogen peroxide. After 10 min of incubation, the plates were read at 492 nm with an ELISA plate reader. The reported values are the mean OD of triplicate.

25 A cut off recognition was calculated for each peptide (mean of the values obtained with HCV negative sera + 3 standard deviations). It allowed to evidence positive responses with 6 out of 44 HCV-positive sera against E1 (amino acids 290-317) (**Figure 8A**), 6 out of 44 against E2 (amino acids 471-500) (**Figure 8B**) and 16 out of 44 against E2 (amino acids 605-629) (**Figure 8C**). Sera A7, A14, A21, A33, A39 and A40 gave a positive signal with the three peptides whereas E2 (605-629) was also recognized by 10 more sera.

30 The presence in the sera of HCV-infected patients of specific antibodies able to react simultaneously with the three regions of E1 and E2 recognized by D32.10 mAb strongly supports their juxtaposition at the surface of circulating enveloped HCV viral particles and their immunogenicity in mice as well as in humans.

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CLAIMS

1. A conformational antibody capable of specifically binding to the natural HCV viral envelope.
2. A conformational antibody according to claim 1, capable of specifically binding to the natural HCV E2 protein.
3. A conformational antibody according to claim 1 or 2, capable of neutralizing HCV infections in patients.
4. A conformational antibody according to any of claims 1 to 3, capable of precipitating the HCV E1E2 complex under its covalent or non covalent forms.
5. A conformational antibody according to any of claims 1 to 4, capable of specifically binding to the natural HCV E1 protein.
6. A conformational antibody according to any of claims 1 to 5, capable of specifically binding to the natural HCV E1 protein, to the natural HCV E2 protein, and of precipitating the HCV E1E2 complex under its covalent or non covalent forms.
7. A conformational antibody according to any of claims 1 to 6, capable of specifically binding an epitope constituted of at least one of the following sequences:
 - amino acids 297 to 306 of HCV protein E1;
 - amino acids 480 to 494 of HCV protein E2;
 - amino acids 613 to 621 of HCV protein E2.
8. A conformational antibody according to claim 7, capable of specifically binding to an epitope constituted of each of the following sequences:
 - amino acids 297 to 306 of HCV protein E1;
 - amino acids 480 to 494 of HCV protein E2;
 - amino acids 613 to 621 of HCV protein E2.

9. A conformational antibody according to any of claims 1 to 8, wherein said antibody is a monoclonal antibody.
- 5 10. A monoclonal antibody according to any of claims 1 to 9, secreted by the hybridoma deposited at the CNCM (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France) on March 19, 2003, under accession number CNCM I-2983.
- 10 11. A monoclonal antibody according to any of claims 1 to 9, secreted by the hybridoma deposited at the CNCM (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France) on March 19, 2003, under accession number CNCM I-2982.
- 15 12. A hybridoma deposited at the CNCM (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France) on March 19, 2003, under accession number CNCM I-2983.
- 20 13. A hybridoma deposited at the CNCM (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France) on March 19, 2003, under accession number CNCM I-2982.
- 25 14. A pharmaceutical composition comprising as active substance at least one of the antibodies of claims 1 to 11 and a pharmaceutically acceptable vehicle.
- 30 15. Use of at least one of the antibodies of claims 1 to 11, for the preparation of a medicament for the diagnostic, the prevention or the treatment of HCV infections.
16. An enveloped viral particle capable of binding to at least one of the antibodies of claim 10 or 11.
17. An antibody which binds to the enveloped viral particle of claim 16.
18. A composition of HCV viral particles derived from initial samples of human blood, plasma or sera, wherein the concentration of HCV RNA copies is about 100 to 1000 fold higher than the concentration of HCV RNA copies in the initial samples of

human, blood, plasma or sera from which it is derived, and is in particular higher than about 10^7 copies/ml.

19. A composition according to claim 18, wherein the number of HCV RNA copies is from about 10^8 to about 10^9 UI per mg of protein.

20. A composition according to claim 18 or 19, wherein the volume of said composition is from about 0.1 ml to about 10 ml.

21. An isolated HCV enveloped subviral particle substantially devoid of HCV RNA and of HCV core protein.

22. An isolated HCV enveloped subviral particle according to claim 21, wherein said subviral particle is liable to bind to any of the antibodies of claims 1 to 11.

23. A composition comprising purified HCV enveloped complete viral particles, said purified HCV enveloped complete viral particles containing HCV RNA, HCV core protein and HCV envelope, and being liable to bind to any of the antibodies of claims 1 to 11.

24. A process for preparing a composition of HCV viral particles comprising the following steps:

- at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;
- resuspension of the HCV enriched pellet in an aqueous solution to obtain a composition of HCV viral particles.

25. A composition of HCV viral particles such as obtained according to the process of claim 24.

26. A process for preparing a composition of HCV enveloped subviral particles comprising the following steps :

- at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;

- resuspension of the HCV enriched pellet in an aqueous solution;
- ultracentrifugation of the resuspended HCV enriched pellet in a sucrose density gradient to separate the elements of the resuspended HCV enriched pellet into fractions according to their density;
- recovery of the fractions containing substantially no HCV RNA, substantially no HCV core protein and containing particles capable of binding to the monoclonal antibody of claim 10 or 11, in particular fractions with a sucrose density of approximately 1.13 to 1.15 g/ml, to obtain a composition of HCV enveloped subviral particles.

27. A composition of HCV enveloped subviral particles such as obtained according to the process of claim 26.

28. A process for preparing a composition of purified HCV enveloped complete viral particles comprising the following steps :

- at least two ultracentrifugations of a sample resulting from a clarified plasmapheresis of a HCV infected patient to obtain a HCV enriched pellet;
- resuspension of the HCV enriched pellet in an aqueous solution;
- ultracentrifugation of the resuspended HCV enriched pellet in a sucrose density gradient to separate the elements of the resuspended HCV enriched pellet into fractions according to their density;
- recovery of the fractions containing from about $5 \cdot 10^5$ to about 10^6 UI of HCV RNA per ml, from about 50 to about 100 pg of HCV core protein per ml, and containing particles capable of binding to the monoclonal antibody of claim 10 or 11, in particular fractions with a sucrose density of approximately 1.19 to 1.21 g/ml, to obtain a composition of purified HCV enveloped complete viral particles.

29. A composition of purified HCV enveloped complete viral particles such as obtained according to the process of claim 28.

30. A process for preparing a monoclonal antibody of claim 10, comprising the following steps:

- immunizing an animal, in particular a mammal, with a composition of HCV viral particles according to claim 18, or such as prepared according to claim 24, and recovering the generated antibodies;
- selecting, among the generated antibodies, monoclonal antibodies on their ability of binding to the HCV viral particles contained in the above mentioned composition of HCV viral particles.

31. A process for preparing a monoclonal antibody of claim 11, comprising the following steps:

- immunizing an animal, in particular a mammal, with a composition of purified HCV enveloped complete viral particles according to claim 23, or such as prepared according to claim 28, and recovering the generated antibodies;
- selecting, among the generated antibodies, monoclonal antibodies on their ability of binding to the purified HCV enveloped complete viral particles contained in the above mentioned composition of purified HCV enveloped complete viral particles.

32. A pharmaceutical composition comprising as active substance the subviral particles of claim 21 or 22, or the composition of claim 27, and a pharmaceutically acceptable vehicle.

33. Use of the HCV enveloped subviral particles of claim 21 or 22, or of the composition of claim 27, to induce an immune reaction against said HCV enveloped subviral particles or against HCV enveloped complete viral particles as defined in claim 22.

34. Use of the HCV enveloped subviral particles of claim 21 or 22, or of the composition of claim 27, for the preparation of a medicament for the diagnostic, the prevention or the treatment of HCV infections.

ABSTRACT

**NEW ANTIBODIES DIRECTED AGAINST HEPATITIS C VIRUS, COMPOSITIONS
OF PARTICLES LIABLE TO BE RECOGNIZED BY SAID ANTIBODIES, AND
5 PHARMACEUTICAL COMPOSITIONS CONTAINING THE SAME**

10 The present invention relates to new conformational antibodies directed against HCV and more particularly to monoclonal antibodies. The invention also relates to compositions of particles liable to be recognized by said antibodies, and to pharmaceutical compositions containing them. The invention also relates to HCV enveloped subviral particles or purified HCV enveloped complete viral particles, and the processes for preparing them.

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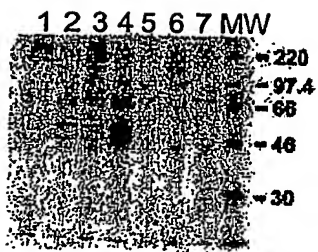


Figure 1A



Figure 1B

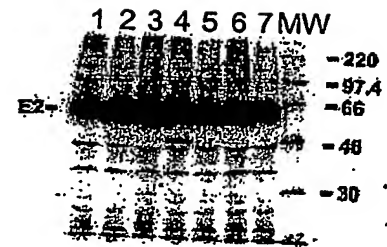


Figure 1C

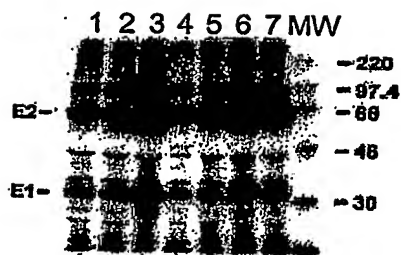


Figure 1D

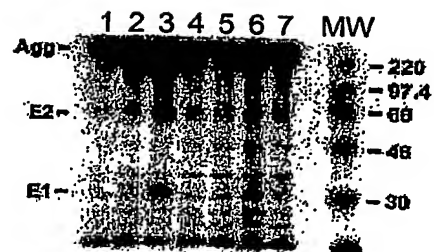


Figure 1E

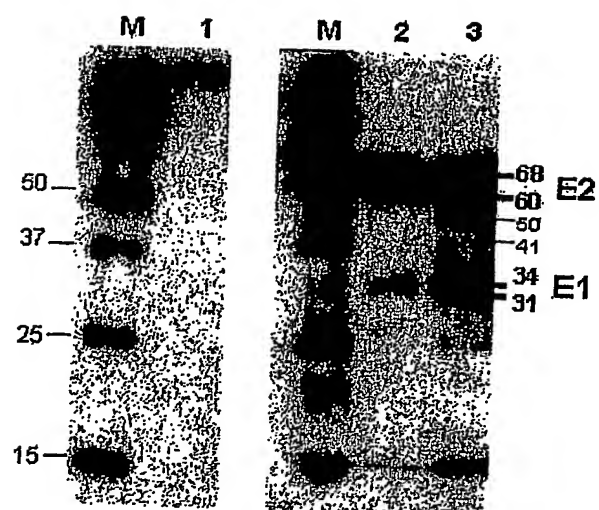


Figure 2

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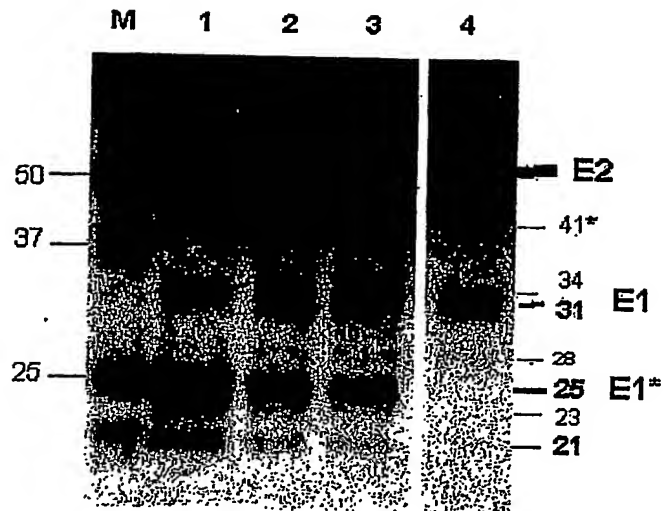


Figure 3A

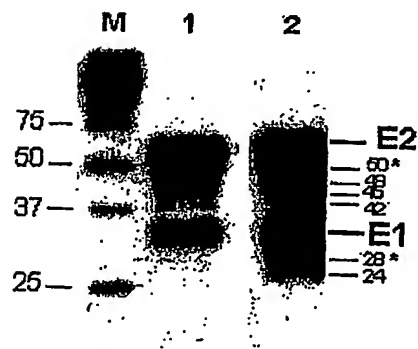


Figure 3B

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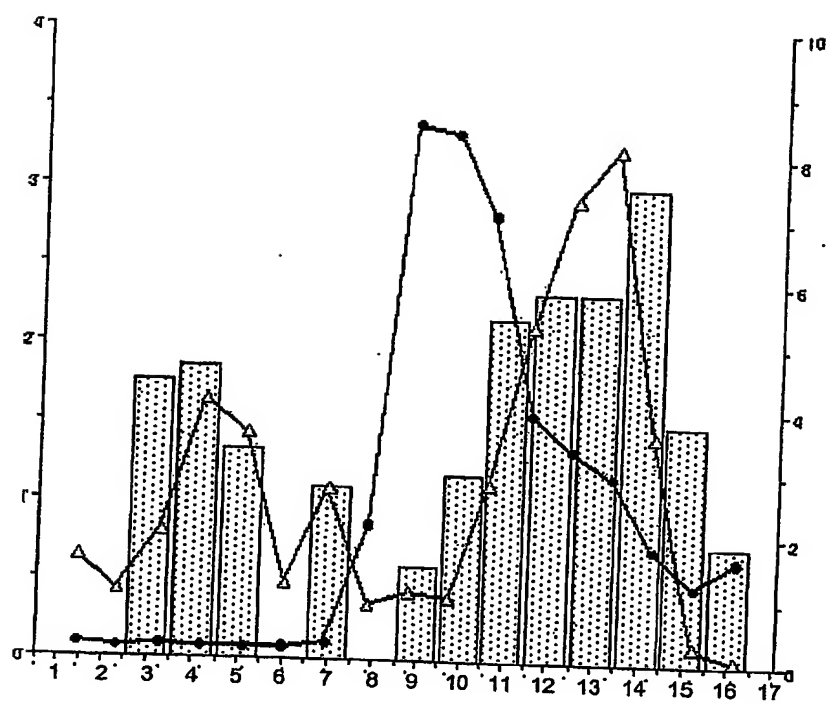


Figure 4

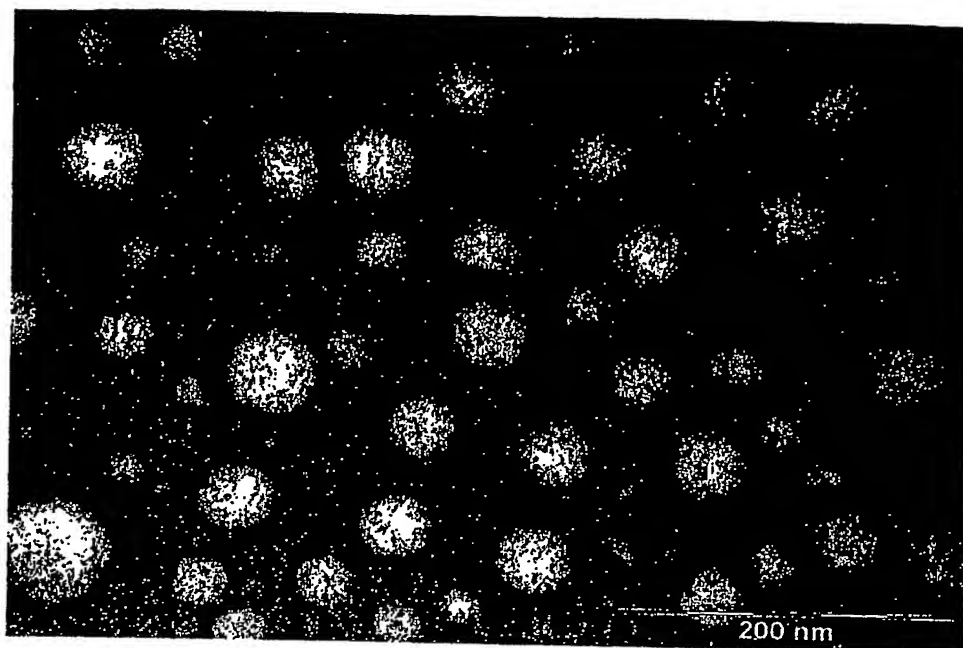


Figure 5A

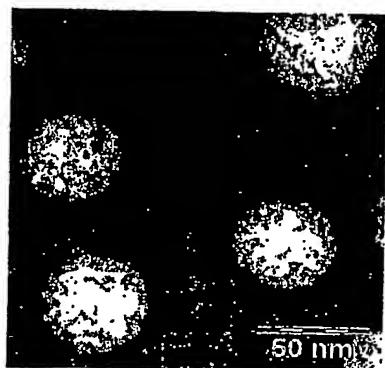


Figure 5B

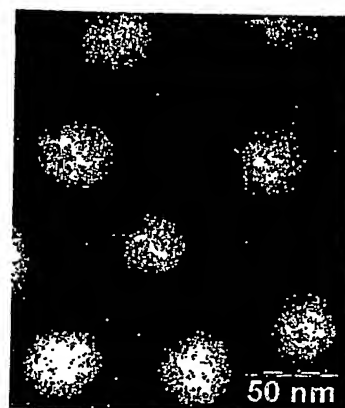


Figure 5C

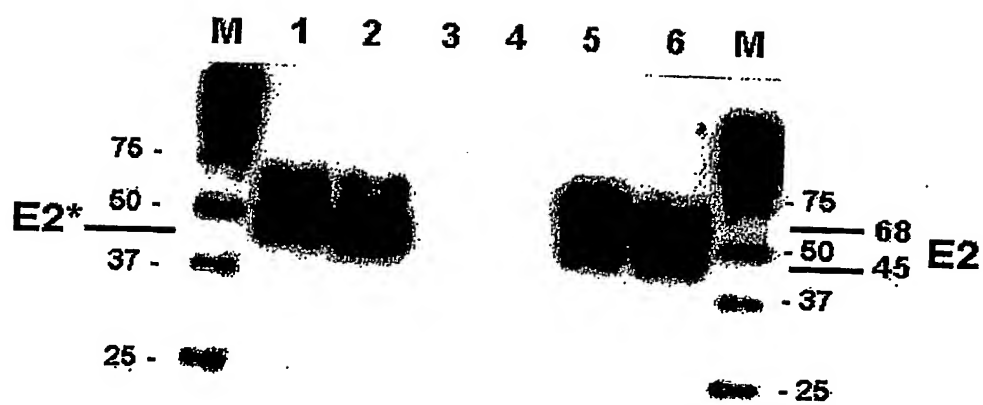


Figure 6

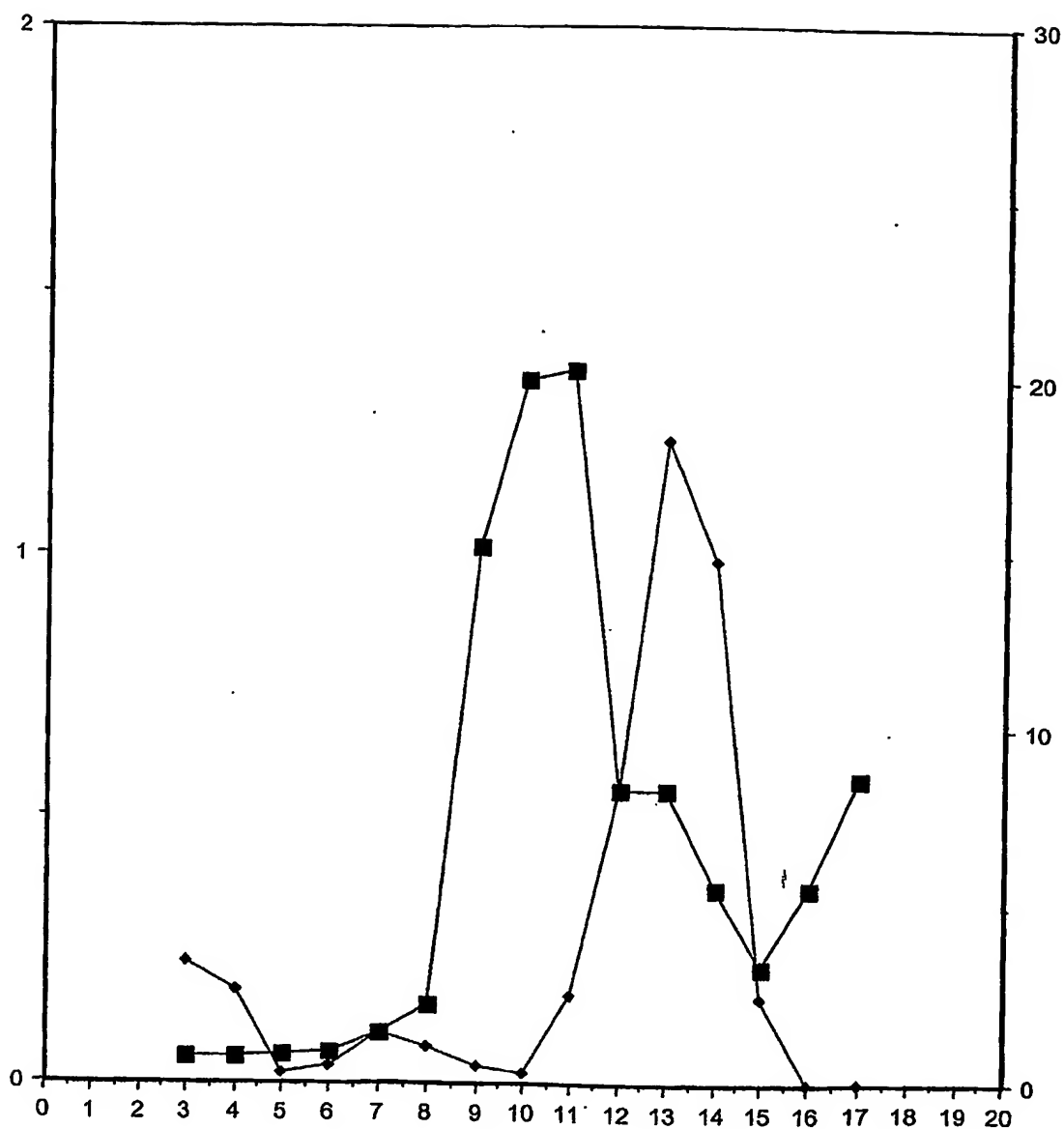


Figure 7

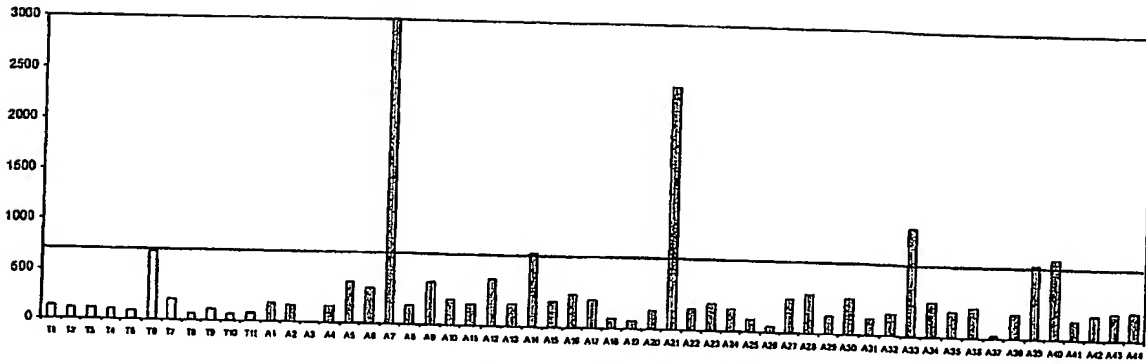


Figure 8A

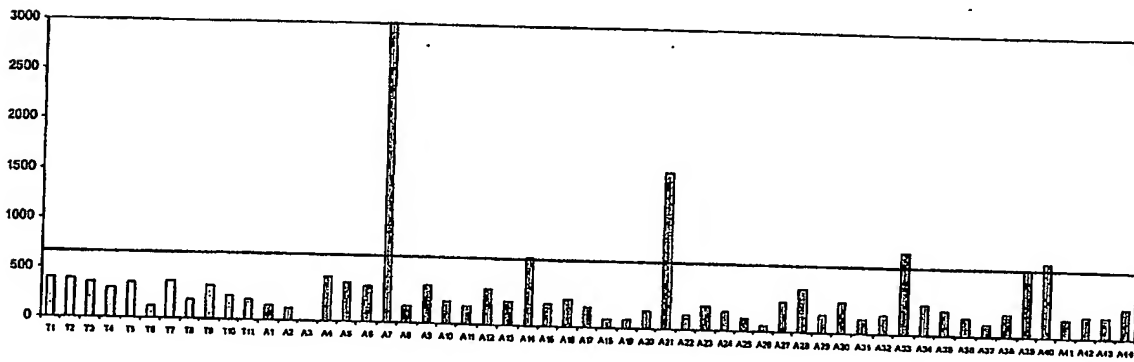


Figure 8B

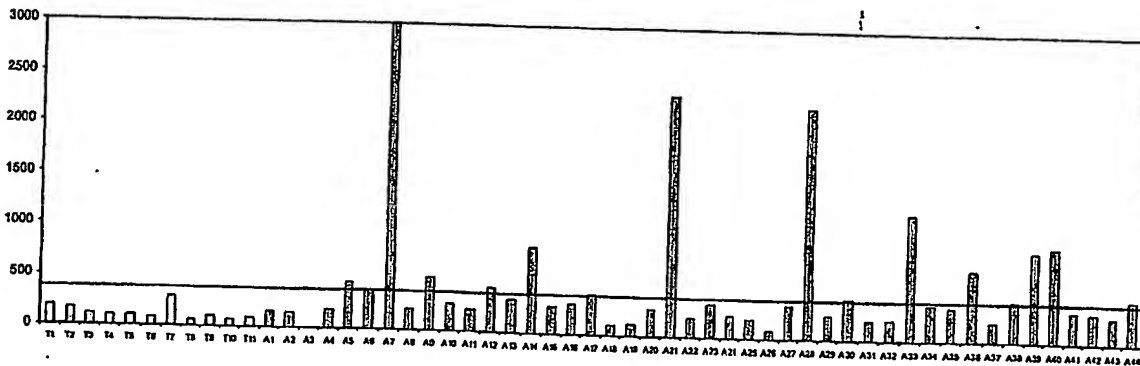


Figure 8C

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